



# Papaya transformed with the *Galanthus nivalis* GNA gene produces a biologically active lectin with spider mite control activity

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## ABSTRACT

Snowdrop (*Galanthus nivalis*) lectin has previously been shown to have anti-feedant and insecticidal activity towards sap-sucking insects. However, its effectiveness against plant-parasitic mites has not been demonstrated. In this study, the commercial papaya (*Carica papaya* L.) cultivar Kapoho, which is highly susceptible to mites, was transformed with the snowdrop lectin (*G. nivalis* agglutinin [GNA]) gene. Polymerase chain reaction confirmed the presence of the transgene and six independent transformed lines were selected for expression analysis. Western blot analysis showed that the lines expressed a recombinant protein with a molecular weight similar to that of the native snowdrop lectin. Leaf extracts containing the recombinant GNA protein agglutinated trypsinized rabbit erythrocytes thus, showing the GNA protein to be biologically active. ELISA and indirect measurement from the agglutination assay showed there to be variation in GNA expression among the lines produced. A laboratory bioassay using carmine spider mites (*Tetranychus cinnabarinus*) suggested improved pest resistance in the transgenic papaya plants. This is the first report that a transgenic plant expressing the GNA gene possesses enhanced resistance to a plant-parasitic mite.

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## 1. Introduction

Papaya (*Carica papaya* L.) is an important fruit crop grown in the tropics and sub-tropics worldwide. It is prone to attack by a number of pests, including aphids, leafhoppers, mites and nematodes. For a review of papaya pests and diseases see Ref. [1]. In Hawaii, the most serious pest damages are caused by leafhoppers, *Empoasca* species [2], and mites including the broad mite, *Hemitarsonemus latus*, the carmine spider mite, *Tetranychus cinnabarinus* Boisd. [3,4] and a relatively new invader, the papaya leaf edgeroller mite, *Calacarus brionese* [5].

Genetic engineering has produced a number of strategies to improve plant resistance to pests and diseases. Papaya transformation was first demonstrated by Pang and Sanford [6] using an *Agrobacterium*-mediated method. The first use of the biolistic gun

approach to transform papaya was in 1990 [7]. Recently, papaya was transformed with the biolistic gun using an insect chitinase gene to demonstrate that genetic engineering can be used for improved pest resistances [8].

Lectins are naturally occurring proteins that possess at least one non-catalytic domain that binds reversibly to a specific mono- or oligo-saccharide [9]. Lectins are ubiquitous, not only in plants, but also in animals, bacteria and fungi. In plants, their role is somewhat an enigma. Recently, in rice (*Oryza sativa*), it was suggested that a mannose-binding jacalin-related lectin may play an important role in rice growth and development [10]. Some lectins have been shown to be toxic to insects and other herbivores [9]. It has been suggested that the anti-insect activity of lectins may be mediated by binding to chitin in the peritrophic matrix or by interacting with glycoproteins on the epithelial cells of the insect midgut. Zhu-Salzman et al. [11] demonstrated that binding of a legume lectin to cell surface receptors in the gut of cowpea bruchid (*Callosobruchus maculatus*) was essential for its anti-feeding activity. More recently, in a study involving rice brown planthoppers (*Nilaparvata lugens*), Du et al. [12] identified ferritin, which is normally involved in iron transport, as the most abundant *Galanthus nivalis* agglutinin (GNA)-binding protein.

GNA, isolated from bulbs of the snowdrop plant [13], is a monocot lectin that has the unique property of recognizing only

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Abbreviations: GNA, *Galanthus nivalis* snowdrop agglutinin; NPTII, neomycin phosphotransferase; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; Bis-Tris-HCl, (bis [2-hydroxyethyl]) imino-tris [hydroxymethyl]-methane-HCl; MES, 2-(*N*-morpholino)ethane sulfonic acid; SDS, sodium dodecyl sulfate; CaMV 35S, cauliflower mosaic virus promoter.

mannose residues. GNA was reported to agglutinate rabbit erythrocytes but to be inactive with human red blood cells [13]. Mannose-recognizing lectins have also been isolated from flowering monocots such as the Chinese daffodil, *Narcissus tazetta* [14] and tulip bulbs, *Tulipa* L. [15]. Although the tulip lectin had a preference for rabbit blood, it was found to agglutinate human red blood cells [15]. The toxicity of many lectins towards higher animals limits their use in crop plants for human consumption. For example wheat germ (*Triticum aestivum*) lectin has strong anti-insect properties but is toxic to mammals and other higher animals [16]. However, GNA is considered to be non-toxic to mammals due to the low binding capacity in the jejunum [17].

Insect-feeding studies have been carried out to investigate the efficacies of a number of different lectins against plant pests. The GNA protein fed in an artificial diet has been shown to be toxic to a number of important homopteran, coleopteran, and lepidopteran insect pests [18]. Gatehouse et al. [19] fed a liquid diet containing pea, potato, wheat germ, concanavalin A and GNA lectins to rice brown planthopper. In that study, GNA was found to be most effective at a concentration of  $1 \text{ g L}^{-1}$ , which resulted in 80% corrected mortality. Researchers have already produced a number of transgenic plants expressing the snowdrop lectin and have tested their resistance against insects. Setamou et al. [20] found that GNA expression in sugarcane (*Saccharum officinarum*) had a negative effect on development and reproduction of the Mexican rice borer (*Eoreuma loftini*). Wu et al. [21] showed that small brown planthoppers (*Laodelphax striatellus*) fed on GNA-transformed rice lines had reduced fecundity, slowed growth and reduced survival. Recently, GNA has been used as a carrier protein in fusion with spider venom toxin to generate toxicity. In artificial diets this fusion protein was found to be toxic to tomato moth, *Lacanobia oleracea* [22] and also to rice brown planthopper and peach potato aphid, *Myzus persicae* [23]. Nagadhara et al. [24] produced rice plants expressing the GNA protein and discovered that they had resistance to all three major sap-sucking pests of rice, namely brown planthopper, green leafhopper, *Nephotettix virescens* [24], and whitebacked planthopper, *Sogatella furcifera* [25].

The proven insecticidal nature of the GNA protein and also the fact that it is non-toxic to higher animals [17] are reasons for using it in an attempt to increase mite resistance in the present study. We hypothesize that papaya plants expressing GNA may have improved resistance to leaf-feeding pests. In this paper, we report the transformation of *C. papaya* (L.) with a gene encoding the snowdrop GNA lectin to produce a biologically active entomotoxic protein and improved resistance to carmine spider mites. This is, to the best of our knowledge, the first report of GNA toxicity to plant-parasitic mites.

## 2. Materials and methods

### 2.1. Plasmid construction

The coding region of GNA was obtained using a polymerase chain reaction (PCR) approach from the plasmid LECGNA2 (provided by E.J.M. Van Damme, Catholic University of Leuven). This plasmid was created by inserting a cDNA fragment into the EcoRI site of pT7T3 18 U [26]. Primers were designed to include an extra BamHI site at the 5' end and a SacI site at the 3' end. The resulting GNA fragment was inserted into a BamHI–SacI digested pBI121 binary vector, a derivative of pBIN19 (Clontech, San Francisco, CA). The plasmid pBI121/GNA contains the neomycin phosphotransferase (*NPTII*) gene as a selectable marker under the control of a nopaline synthase (Nos) promoter and terminator. GNA was under the control of a constitutive CaMV 35S promoter and a second Nos 3' terminator.

### 2.2. Plant transformation

A biolistic gene gun was used to introduce the plasmid pBI121/GNA DNA into the papaya cultivar Kapoho, using a modified method adapted from Fitch et al. [7]. Twenty plates of embryogenic calli derived from Kapoho were used for transformation. Bombarded embryogenic calli were transferred and selected on media containing  $100 \text{ mg mL}^{-1}$  geneticin in the brand name product G418. After 3–4 months, the calli surviving on medium containing G418 were regenerated into plantlets. The medium used for maintenance of tissue culture material was  $4.3 \text{ g L}^{-1}$  MS salts,  $30 \text{ g L}^{-1}$  sucrose,  $0.1 \text{ g L}^{-1}$  myo-inositol,  $1 \text{ mL L}^{-1}$  MS vitamin IV, pH 5.7. For shoot production  $0.1 \text{ mg L}^{-1}$  naphthaleneacetic acid (NAA) and  $0.1 \text{ mg L}^{-1}$  benzyladenine (BA) were added. To promote rooting of tissue cultured plants,  $0.2 \text{ mg L}^{-1}$  indole-3-butyric acid (IBA) was added into the shoot media. Selected transformants were transferred onto rooting media until roots formed and subsequently transferred into potting soil (Sunshine 4 mix, United Agricultural Supply Company). Potted plants were maintained in a greenhouse under natural lighting where temperatures ranged between  $22^\circ\text{C}$  and  $26^\circ\text{C}$ .

### 2.3. Polymerase chain reaction and quantitative reverse transcriptase (RT)-PCR analysis

Fully-expanded leaves of approximately 6-month-old, greenhouse-grown papaya plants were collected, immediately frozen in liquid nitrogen, and stored in a  $-80^\circ\text{C}$  freezer until extracted. Genomic plant DNA was isolated using the method of Dellaporta [27]. The primers used to amplify a 700 bp fragment of the *NPTII* gene were 5'-AGAGGCTATTCGGCTATGAC-3' and 5'-GTCAA-GAAGGCGATAGAAGG-3'. A 500 bp GNA fragment was amplified using 5'-ATGGCTAAGGCAAGTCTCTC-3' and 5'-TCATTACTT-TGCCGTCACAAG-3'. As a control, primers for actin were also used, 5'-ACTACGAGTTGCTGATGA-3' and 5'-AACCACCACTGAGCA-CAATG-3'. The actin primers, based on papaya actin partial sequence (accession AY906938), amplified a 200 bp fragment. Both GNA specific and actin primers were used at  $1 \mu\text{M}$  concentration. A total volume of  $50 \mu\text{L}$  was prepared for use in a Bio-Rad iCycler<sup>®</sup> thermocycler. The conditions were  $95^\circ\text{C}$  for 2 min followed by 30 cycles at  $95^\circ\text{C}$  for 30 s,  $60^\circ\text{C}$  for 30 s,  $72^\circ\text{C}$  for 45 s with a final extension at  $72^\circ\text{C}$  for 10 min.

Total RNA was extracted from the same leaf samples used for PCR according to the method of Verwoerd et al. [28]. First strand cDNA synthesis was carried out using an oligo dT primer and ImProm-II<sup>™</sup> reverse transcriptase (Promega). Platinum<sup>®</sup> SYBR<sup>®</sup> Green qPCR Supermix UDG (Invitrogen) was used for the PCR step. An Opticon 2 DNA engine (MJ Research) was used for real-time quantification of PCR products. For quantitative PCR, the same actin primers and GNA/*ce:italica* specific primers, 5'-TTTGGCCGCCATCTTCCTTG-3' and 5'-TGGCCTCCAGTGTGCTTG-3', amplifying a product size of 256 bp, were used. DNase-treated total RNA (RQ1, Promega) (364 ng) was used for the reverse transcription reaction and 18 ng of cDNA was used for subsequent PCR amplification. The above amplification protocol was used for standard PCR. As a control, RNA samples without RT (RT minus) were included to be certain that the RNA samples were not contaminated with genomic DNA.

### 2.4. Western blot analysis

Fully-expanded leaves of approximately 6-month-old, greenhouse-grown papaya plants were collected. Samples were ground in liquid nitrogen and homogenized in  $1\times$  phosphate-buffered saline (PBS) pH 7.4, and the supernatant was collected after centrifugation at  $10,000\times g$  for 10 min. Total soluble protein

concentration was determined using bovine serum albumin (BSA) as a standard in a Bradford assay [29].

Protein samples were separated under denaturing conditions on Nupage™ 4–12% BisTris (Bis [2-hydroxyethyl] imino-tris [hydroxymethyl]methane)-HCl gels (Invitrogen) using MES SDS (2-(*N*-morpholino) ethane sulfonic acid, sodium dodecyl sulfate) running buffer. A Westernbreeze chemiluminescent immunodetection system (Invitrogen) was used for detecting the GNA protein. Goat anti-GNA (Vector Laboratories) was used as the primary antibody, with a secondary antibody specific to goat and procedures were as outlined by the manufacturer. Bands were visualized by exposure to blue sensitive autoradiographic paper (Marsh Bioproducts Inc.).

### 2.5. Recombinant GNA protein analysis

An agglutination assay was used to check for the biological activity of the recombinant GNA protein. The method used was adapted from Cammue et al. [15] and from E.J.M. Van Damme (personal communication). Plant extracts were prepared as described for Western blotting. Total soluble protein was determined by a Bradford assay [29]. Rabbit erythrocytes (provided by Dr. David Clements, Hawaii Biotech Inc.) were prepared by six washes with 1× PBS pH 7.4 and 7 min centrifugation at  $300 \times g$ . A 10% (v/v) solution of cells was trypsinized by adding  $1 \text{ mg mL}^{-1}$  trypsin and then incubated at  $37^\circ\text{C}$  for 1 h. Ten microlitres of crude plant extract, with concentration approximately 3–6  $\mu\text{g}$ , was placed in each well of a 96 U-walled microtiter plate. Ten microlitres of 1 M ammonium sulfate was added per well to reduce the pH. Finally, 30  $\mu\text{L}$  of a 2% solution (v/v) of trypsinized erythrocytes was added and the plate incubated at room temperature for an hour. Clumps of red blood cells were checked visually, photographed, and the time noted when they appeared.

### 2.6. ELISA assay

Plant extracts were prepared in 1× PBS pH 7.4 as described for Western blotting. A Bradford assay [29] was used to determine total soluble protein. For ELISA, 40  $\mu\text{g}$  of total protein was brought up to a total volume of 100  $\mu\text{L}$  in 1× PBS. The protein was bound to the wells of an Immulon 2HB plate (Dynex Technologies Inc.) by overnight incubation at  $37^\circ\text{C}$ . Three replicates per sample were included. The plate was blocked for 1 h at room temperature with 200  $\mu\text{L}$  1× PBS-Tween (0.05% Tween-20) with 0.5% BSA. The wells were washed three times with 1× PBS-Tween and then the plate was incubated for 2 h at room temperature with Goat anti-GNA (Vector Laboratories) primary antibody (1:1000 dilution in 1× PBS-Tween). Wells were washed three times with 1× PBS-Tween and the plate was incubated with a secondary antibody specific to goat for 2 h (from Westernbreeze chemiluminescent immunodetection system, Invitrogen). Wells were again washed and 100  $\mu\text{L}$  pNPP (*p*-nitrophenyl phosphate disodium salt) substrate (Sigma) was added. After 1 h the reaction was stopped by addition of 50  $\mu\text{L}$  3 M NaOH and absorbance at 405 nm was read in a MRX plate reader (Dynatech Laboratories). A standard curve was produced using dilutions of purified GNA protein (Vector Laboratories) and this was used to calculate  $\mu\text{g/mL}$  of GNA in the samples and also the percentage of total soluble protein which the GNA represented.

### 2.7. Mite bioassay

A laboratory bioassay examined the dynamics of carmine spider mites feeding on leaves from transgenic papaya plants. A cork borer (3 cm diameter) was used to cut disks from leaves of field-

grown plants, approximately 1-year old. For each plant to be tested, six disks were prepared from multiple leaves. For the control, Kapoho, multiple trees were sampled but for the transformed lines only one tree per line was available. Disks were cut to include the midvein because mite populations, under natural conditions, were found to be higher in the center of leaves. Leaf disks were surface sterilized by soaking in 10% bleach solution for 2 min and then rinsed in sterile water. Disks were floated abaxial side uppermost on sterile distilled water in 9 cm Petri plates. Water was used to prevent mites moving between disks and also to prevent the disks from desiccating. Using a fine artist paintbrush, two adult mites collected from field-grown Kapoho trees, were placed on each disk. Adult mites were chosen because they are visible to the naked eye, whereas juvenile stages are not. Active adults of similar size were selected for uniform infestation. Plates were maintained under artificial lights at room temperature ( $20\text{--}24^\circ\text{C}$ ).

One week after mites were placed on the disks, the number of unhatched eggs and juveniles produced from hatched eggs were counted using a dissecting microscope (Stemi SR, Zeiss, West Germany).

Chlorophyll of each disk was extracted 14 days after mite infestation and used as a measure of leaf damage. Each disk was ground separately in 80% acetone, the resulting solution was centrifuged for 1 min at 14,000 rpm in a bench top centrifuge, and the supernatant was removed. Chlorophyll was quantified with a Beckman, Du-70 spectrophotometer with readings taken at wavelengths of 645 and 663 nm. Chlorophyll *a* content was calculated from  $A_{645} - 0.1994 A_{663}/43.75$  and chlorophyll *b* was calculated from  $A_{663} - 9.27 \text{ chlorophyll } b/84.02$ , according to the method of Arnon [30]. Total chlorophyll was determined by addition of chlorophyll *a* and *b* divided by the leaf area and presented as  $\mu\text{g}$  chlorophyll/ $\text{cm}^2$  leaf.

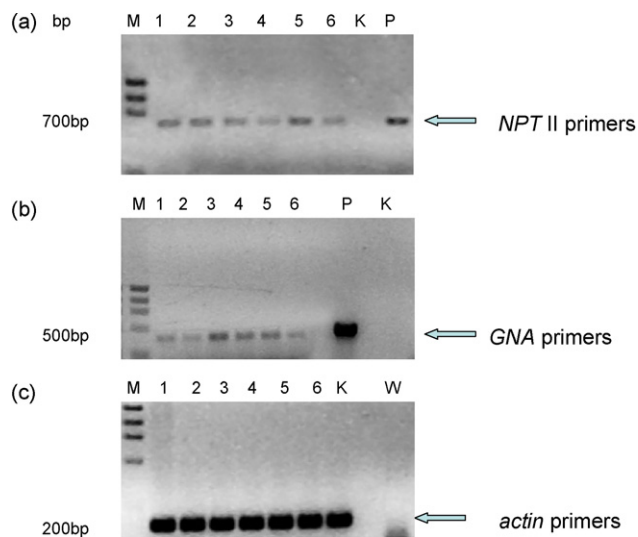
## 3. Results

### 3.1. Transformation of papaya with GNA gene

Sixty putative transformants were selected from 20 bombarded plates after 3–4 months with monthly subculturing on selection medium containing geneticin ( $100 \text{ mg L}^{-1}$ ). Selectively grown callus on G418 medium was screened for the presence of the *NPTII* gene using PCR. Leaves of regenerated plants were tested for the presence of both *NPTII* and *GNA* using specific primers with PCR (Fig. 1a and b). Water (W) and wild-type Kapoho (K) were used as negative controls and plasmid DNA (P) was used as positive control. Wild-type Kapoho DNA spiked with plasmid DNA was also checked to avoid false negative results (not shown). Primers for actin were used as an internal control as it is a single copy endogenous gene (Fig. 1c). No fragment was amplified in the wild-type Kapoho sample with *NPTII* and *GNA* primers (Fig. 1a and b), while actin primers amplified a 200 bp product from all plants (Fig. 1c). Of the 60 putative transformants, PCR showed that 19 (32%) were positive for *NPTII* gene and 14 of these were positive also for the *GNA* gene. Transformation efficiency was calculated as 0.7 PCR positive lines per bombarded plate (14 lines out of 20 plates). This frequency corresponds with our earlier work in which we reported approximately one transformed line per bombarded plate using a similar selection methods [31,32]. The 14 transgenic lines, giving positive results with both *NPTII* and *GNA* genes, were selected for further growth. None of these 14 lines showed any visual phenotypic modification. Six were then chosen for a more detailed study of recombinant protein expression.

RT-PCR analysis was carried out to check for the expression of the *GNA* transgene in the six selected PCR positive lines along with



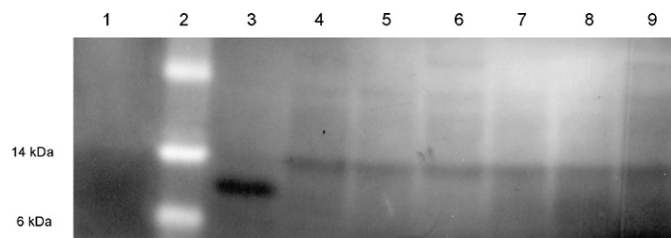


**Fig. 1.** Molecular analysis of GNA transgenic lines. DNA was extracted from fully-expanded leaves of approximately 6-month-old, greenhouse-grown papaya plants and used in PCR analysis. (a) *NPTII* primer set with product of ~700 bp. (b) *GNA* primer set with product of ~500 bp. (c) Actin primer set with product of ~200 bp. Lanes: M molecular weight marker ( $\Phi$ X174 DNA-*Hae* III digest, New England Biolabs), 1–6 transgenic papaya lines, P plasmid positive control, K wild-type Kapoho control, W water negative control.

Kapoho as a non-transformed control. RT-PCR products obtained using *GNA* and actin primers were visualized by agarose gel electrophoresis and gels were similar to Fig. 1b and c (RT-PCR gels not shown). The RT-PCR results showed that the *GNA* gene was amplified by PCR after reverse transcription using total RNA extract in six transformed lines, but absent in non-transformed and in RT minus (no RT) controls, indicating the presence of the *GNA* gene and transcript in these selective transgenic lines.

### 3.2. Recombinant GNA protein analyses

The recombinant GNA protein in the papaya leaves was detected by Western blotting analysis. All six transgenic lines positive for PCR expressed GNA in their leaf tissues (Fig. 2). Lines which had given negative results for PCR were also negative for this test (results not shown). Purified GNA protein showed a single band at 11 kDa. All six transgenic lines showed bands at 13 kDa

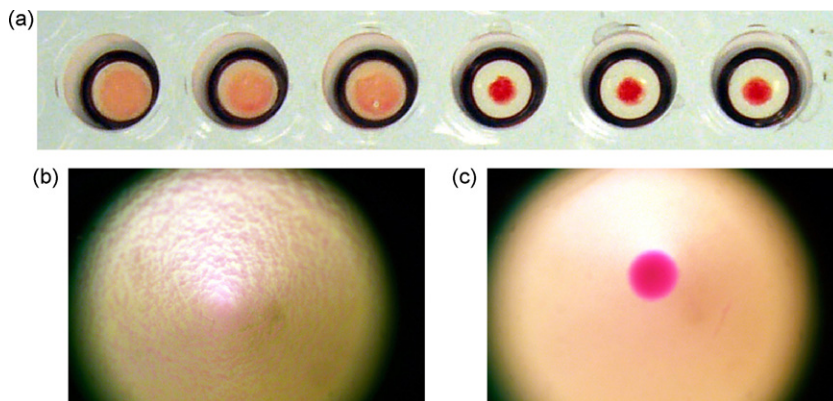


**Fig. 2.** Western blot analysis of recombinant GNA protein expression in six transformed papaya plants. Lanes: (1) wild-type Kapoho (negative control), (2) protein standard (see Blue<sup>®</sup> Plus 2, Invitrogen), and (3) wild-type Kapoho spiked with 20 ng purified GNA protein (positive control), 4–9 transgenic papaya lines. Five micrograms of total protein were loaded per lane.

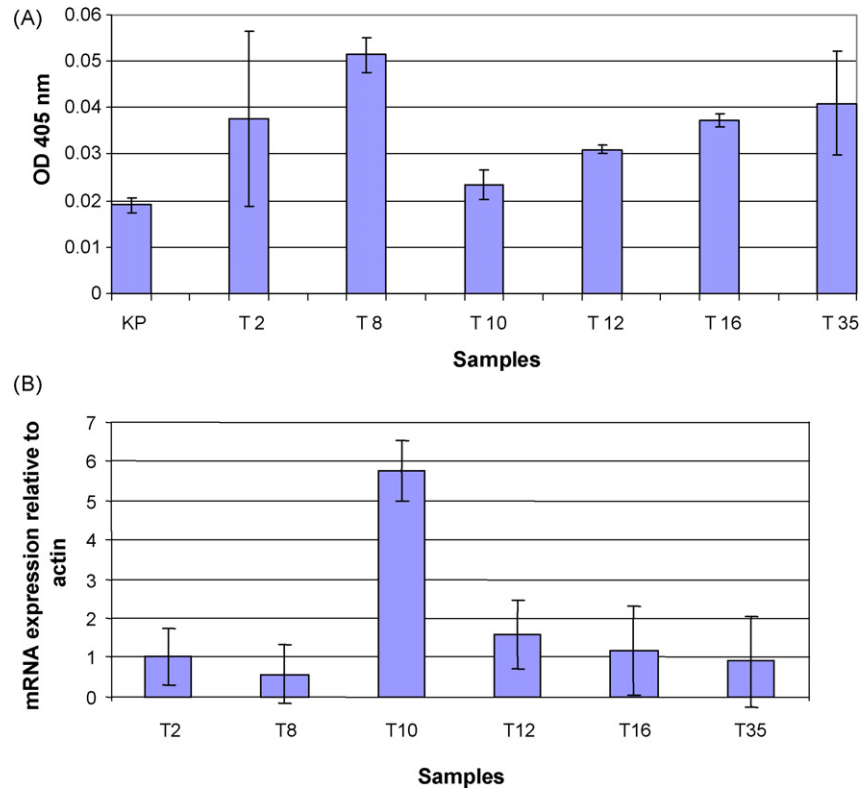
(Fig. 2). The recombinant GNA proteins had a slightly higher molecular weight than the purified GNA standard protein. This result indicated that the *GNA* transgene was correctly transcribed and expressed in transformed lines, but that the GNA proteins were slightly modified during post-translation process [13].

Functional integrity of this recombinant protein was tested in an agglutination assay in which crude protein extracts from leaf tissue were tested for their ability to agglutinate rabbit erythrocytes. Twenty-eight lines including lines that had previously given negative PCR results were also tested. Only lines positive for PCR analysis gave positive results in this assay. Fig. 3 shows an example of the results from the agglutination assay. A positive reaction was recorded if there was a uniform coating of the bottom of the well by erythrocytes. In a negative reaction there was a central button of erythrocytes surrounded by a concentric clear zone devoid of erythrocytes. Trypsinized rabbit erythrocytes were used for this assay as it has previously been shown that the sensitivity of erythrocytes to agglutination is enhanced by treatment with a protease. For the GNA protein, Van Damme and Peumans [13] determined that the minimal concentration of lectin required to agglutinate rabbit erythrocytes was  $1.8 \mu\text{g mL}^{-1}$  or  $0.8 \mu\text{g mL}^{-1}$  for untreated and trypsinized cells, respectively. In the present study, the highest concentration to agglutinate trypsinized rabbit erythrocytes was found to be  $0.625 \mu\text{g mL}^{-1}$ . This is consistent with the level of expression determined in the crude leaf extracts and with the known activity of pure GNA protein.

The six selected lines all gave positive results for the agglutination assay, indicating that the recombinant GNA protein



**Fig. 3.** Photographs of the agglutination assay used to determine biological activity of the recombinant GNA protein. Thirty microlitres of a 2% solution of trypsinized rabbit erythrocytes was incubated for 1 h at room temperature with crude leaf extracts (3–6  $\mu\text{g}$ ) to test for agglutination. (a) U-shaped microtiter plate with positive and negative reactions. A positive reaction has a uniform coating of the bottom of the well by erythrocytes. A negative reaction shows a central button of erythrocytes surrounded by a concentric clear zone devoid of erythrocytes. (b) Higher magnification of a single well showing a positive reaction. (c) Higher magnification of a single well showing a negative reaction.



**Fig. 4.** Comparison of relative mRNA levels and GNA expression in transformed papaya lines, T2, T8, T10, T12, T16 and T35. (a) Determination of GNA expression using ELISA. Three replicate assays were set up using a single crude extract from pooled leaf material. A standard curve was created using serial dilutions of purified GNA protein and used to determine GNA content of the samples. (b) mRNA levels were measured by quantitative RT-PCR relative to actin. Figures are for six replicates. Error bars represent standard deviation.

is biologically active. An indirect calculation of the GNA protein content was made using the assay results. The agglutination titer was calculated and compared to the titer of a purified GNA protein of known concentration. The percentage of total soluble protein that the GNA protein represented was calculated. From the agglutination assay, recombinant GNA expression was found to be between 0.03% and 0.74% of total soluble protein extracted from leaf tissue (results not shown).

An ELISA assay was used to further examine recombinant GNA protein expression (Fig. 4a). Using ELISA to determine protein expression calculated lower amounts than the amount calculated indirectly with the agglutination assay. Elisa figures for GNA expression ranged from 0.02% to 0.05% of total soluble protein extracted from leaf tissue. Lines which had previously been shown to have the highest level of GNA expression in the agglutination assay were also the highest in the ELISA. It was found that there was no significant difference between Kapoho and the lines with the lowest GNA expression, T10 and T12 (Statistix 7.0 software). For lines T8, T16 and T35, however, there was a significant difference between each and the control Kapoho ( $p < 0.05$ ).

Quantitative RT-PCR analysis was carried out to determine mRNA levels of the GNA transgene using total RNA from the six selected PCR positive lines along with Kapoho as a non-transformed control (Fig. 4b). The relative mRNA levels were calculated using actin for normalization and compared with GNA protein levels based on the agglutination assay. Generally, there was no significant correlation between the mRNA and protein expression, indicating post-transcription or translation modification or variable protein turnover of GNA in different transgenic lines.

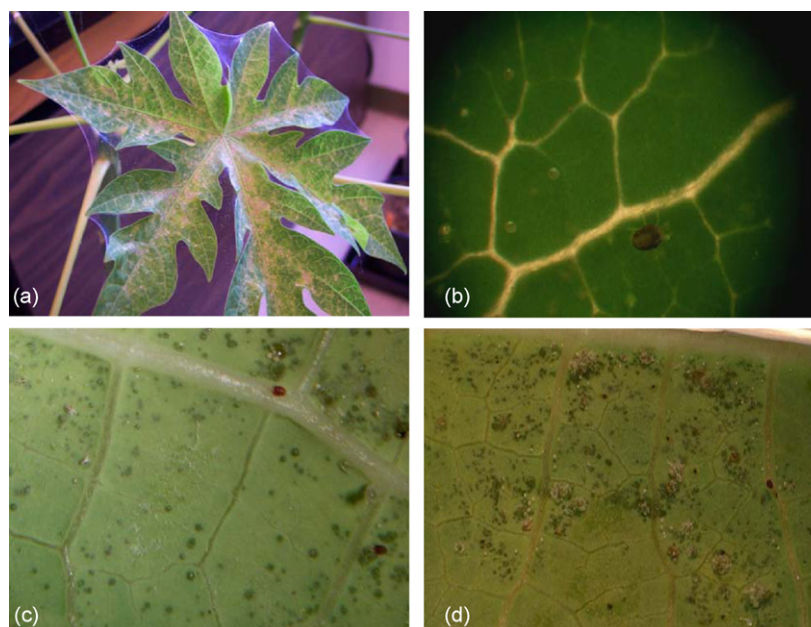
### 3.3. Mite bioassay

A bioassay using carmine spider mites (*T. cinnabarinus*) was undertaken to examine any difference in resistance between GNA-expressing plants and non-transformed Kapoho control. Photographs of papaya leaves colonized and damaged by carmine spider mites are shown in Fig. 5. Counts of unhatched eggs produced and hatched juveniles counted along with chlorophyll extracted from the leaf disks are shown in Table 1.

The mite population was examined 7 days post-inoculation. Based on the ELISA result, lines T8, T16 and T35, with highest GNA expression, were grouped together as a transformed group. This allowed for replication as only one tree per transformed line was sampled.

A significant difference was found (Statistix 7.0 software) between the number of eggs produced by mites feeding on leaves of non-transformed Kapoho or on leaves of the GNA-expressing lines. The highest number of unhatched eggs was on Kapoho while the transformed lines had considerably fewer eggs. However, there was no significant difference in the number of juveniles between the GNA-expressing lines and Kapoho ( $t$ -test, Statistix 7.0 software). If the number of unhatched eggs and the number of hatched juveniles are added to indicate the total reproductive capacity of mites feeding on each line, there was a significant difference between the transformed lines and Kapoho ( $p < 0.05$ ).

Loss of chlorophyll, extracted 14 days post-infestation, was used as a measure of leaf damage caused by mite feeding (Fig. 5a). Mite larvae, nymphs, and adults all feed by puncturing cell walls and sucking cell contents, particularly that of the chloroplasts. Killing of individual cells or groups of cells by feeding mites



**Fig. 5.** Photographs of papaya leaves infested with carmine spider mite (*Tetranychus cinnabarinus*). (a) Papaya leaf showing yellowing of leaves due to spider mite feeding and webbing around leaf margins which is used for transport. (b) Adult spider mite with eggs on papaya leaf (5× magnification). (c) GNA-expressing leaf disk showing sparse feeding damage (2× magnification). (d) Kapoho leaf disk showing more concentrated and heavier feeding damage (2× magnification).

**Table 1**  
Papaya leaf disk assay to examine the effect of feeding on lines expressing a recombinant GNA protein on survival and egg production of *Tetranychus cinnabarinus*

Sample	Mean number unhatched eggs	Mean number hatched eggs (seen as juveniles)	Measure of reproductive capacity	Chlorophyll extracted from leaf disk ( $\mu\text{g}/\text{cm}^2$ )
Kapoho	33.0 <sup>a</sup>	1.0 <sup>a</sup>	34.0 <sup>a</sup>	0.79 <sup>a</sup>
Pooled transformed lines	8.0 <sup>b</sup>	2.0 <sup>a</sup>	10.0 <sup>b</sup>	1.15 <sup>b</sup>

Data shown are for six experimental replicates. Based on ELISA analysis of GNA expression, lines T8, T16 and T35 were shown to be significantly different to Kapoho and were pooled. 1-Week post mite infestation, the number of unhatched eggs produced and juveniles which had emerged from eggs were counted. The sum of the number of unhatched eggs and juveniles indicates the potential number of reproductive adults and thus, is a measure of reproductive capacity. Chlorophyll was extracted 2 weeks postmite infestation as an indicator of leaf disk damage. Statistix 7.0 software was used to perform a *t*-test to compare the mean values of the pooled transformed lines with that of Kapoho. Different letters indicate significant differences ( $p < 0.05$ ).

produces the transparent, yellow, or tan patchwork of damage that indicates the level of mite infestation (online resource, [nathist.sd-state.edu/orchids/Pests/mites.htm](http://nathist.sd-state.edu/orchids/Pests/mites.htm)). Kapoho appeared to have the greatest number of eggs and individual mites and the lowest amount of chlorophyll. Thus, the damage to the Kapoho leaf disks was greatest. It appeared that the mites fed in a more concentrated area on the Kapoho leaf disks than on the transformed plant leaves. Once they began feeding in one area they would remain fairly stationary and continue to feed. Mites appeared to spend more time traveling across the GNA-expressing leaf disks and would feed sporadically as they traversed the disk. While this resulted in more widespread feeding damage, the actual total area of damage was smaller (Fig. 5c and d).

It was calculated from the agglutination assay that individual GNA lines expressed different amounts of GNA protein. T8 and T16 had the lowest amount while T35 had the highest concentration. It appears that there was a positive relationship between the concentration of GNA and mite fecundity. The higher the GNA in the leaf disk, the fewer eggs produced. At the lower level of 0.03% lectin (T8 and T16), there was a threefold reduction in egg production. For T35 there was an eightfold reduction (0.74% GNA). Line T35 also had the lowest number of juvenile mites.

#### 4. Discussion

We report the production of transgenic papaya plants expressing the snowdrop lectin, GNA, under a constitutive promoter. GNA expression levels in selective transgenic lines were determined using quantitative RT-PCR and an ELISA assay. GNA expression in transgenic plants has been quantified previously using a variety of different methods. Levels reported appear to depend on the promoter used to control GNA expression and possibly the quantification methods employed. Generally, the quantities obtained in the present study were comparable to those obtained in other plant species using the same constitutive promoter. For example, Gatehouse et al. [19] used a construct with CaMV 35S promoter driving expression of GNA in tobacco (*Nicotiana* spp.) and quantified the level using a quantitative dot-blot immunoassay with an anti-GNA antibody. Those workers reported GNA levels of up to 1% total protein in primary transformants. Ripoll et al. [33], also using CaMV 35S promoter but in *Arabidopsis thaliana*, estimated GNA levels from Western blotting to be from 0.1% to 1.3%. Saha et al. [34] introduced a lectin from garlic leaves (*Allium sativum*) into rice (*O. sativa*) and quantified lectin expression using ELISA to be between 0.26% and 0.72% total soluble proteins. Shah et al. [35] introduced GNA into wheat under a constitutive

ubiquitin 1 promoter and found expression of GNA to be 0.05% of total soluble protein using Western blotting. Again using a constitutive ubiquitin promoter to control GNA expression Setamou et al. [20] produced transgenic sugarcane and estimated GNA expression to be 0.89% from Western blotting. Wang et al. [36] put GNA into maize (*Zea mays* L.) under the control of a phloem-specific promoter and found GNA expression to be between 0.13% and 0.28% based on immunoblot staining intensity.

The effectiveness of constitutive promoters versus tissue-specific ones to control GNA expression also differs. For example, Saha et al. [37] produced rice plants expressing an agglutinin (ASAL) from leaves of garlic (*A. sativum*) with different promoters, including two phloem-specific promoters, *rolC* from *Agrobacterium tumefaciens* and rice sucrose synthase-1 (RSs1), and a constitutive CaMV 35S promoter. When they quantified ASAL in transgenic rice using ELISA, they found that plants with CaMV 35S promoter had the highest expression (0.86–1.01% of total soluble protein) followed by *rolC* (0.38–0.52%). RSs1 had the lowest expression (0.11–0.31%). Shi et al. [38] also used a CaMV 35S promoter and the phloem-specific RSs1 promoter to control GNA expression in tobacco plants. They compared densitometer readings of immunoblots and found that GNA expression driven by the RSs1 promoter was only 0.02–0.04% of the level given by the CaMV 35S promoter. However, histochemical staining showed that within phloem cells the RSs1 promoter was comparable in activity to the CaMV 35S promoter. These findings highlight the importance of targeting transgene expression. If a sap-sucking pest such as aphids in the study by Shi et al. [38] is targeted, then a phloem-specific promoter would be preferable. However, with a pest such as mites, which are not limited to phloem feeding, using a constitutive promoter may be more effective. Although tissue-specific promoters have their merit, in three cases it was found that a constitutive promoter was more effective when compared with a tissue-specific one [39]. In the present study, we used a constitutive promoter with the aim of improving plant resistance to pests both above and below the soil.

The agglutination assay using crude leaf extracts from the GNA-expressing lines to agglutinate trypsinized rabbit erythrocytes showed that the recombinant GNA protein is biologically active and behaves similarly to the native one. The amount of GNA was estimated by comparing the agglutination titer (reciprocal of the highest dilution at which the extract still agglutinates red blood cells after incubation for 1 h at room temperature) against that of solutions of known GNA concentration. Using this indirect calculation gave higher estimates of GNA than calculated from ELISA which we assume is more accurate. Using both methods, the same lines had the highest amount of GNA ( $\mu\text{g mL}^{-1}$ ).

The transgenic plants produced in this project expressed different levels of the GNA protein, presumably due to some combination of differences in transcriptional activity at the sites of insertion in the chromosome, copy number of the transgene, and post-translational modifications. We found that mRNA levels of GNA were not correlated with the protein levels in different transgenic events, indicating post-transcriptional differences, or protein modification. That protein modification might account for the lack of correlation between mRNA and protein levels is supported by Western blot analysis showing that the recombinant GNA proteins were slightly larger than the purified GNA protein. However, differences in protein turnover, due to different insertion sites, might also explain the poor correlation between mRNA and protein. In previous work, Ripoll et al. [33] found that GNA expression could protect *A. thaliana* from root-knot nematodes, but greatest resistance was not necessarily linked to the highest GNA expression. This is not the case for the transformed plants produced in this study. For example, line T35, which by ELISA

was calculated to have the second highest level of GNA protein expression, was found in a laboratory test to have the lowest mite population (result not shown). Meanwhile, line T8 which had the highest protein level as measured by ELISA and agglutination assay and also the lowest mRNA level, was found to have a higher number of mite eggs and more leaf damage (result not shown). It would be interesting to see if this result was obtained in a field trial.

Recent work by Saha et al. [37] highlighted a novel approach in using lectins to provide resistance against phloem-limited viruses. They suggested that incorporating a lectin from garlic into rice plants not only conferred resistance to the green leafhopper, which is a vector for tungro viruses, but also provided resistance against disease caused by these viruses. In an earlier report, Bannerjee et al. [42] identified in the gut of the mustard aphid (*Lipaphis erysimi*) a receptor protein that has binding affinity to ASAL and also plays a role in aphid-mediated virus transmission. Papaya is prone to a ringspot virus for which aphids act as a vector. It would be interesting to see if GNA-expressing papaya had an anti-feedant effect on aphids and thus, reduced spread of the virus.

One concern regarding transgenic crop plants expressing entomotoxic proteins is that the plants may be toxic to non-target insects. Much recent work carried out with GNA has been to address this concern. Researchers have examined the effect on insects which feed on GNA-expressing plants. In one such study, Peumans et al. [43] identified high levels of a lectin in the nectar of leek flowers (*Allium porrum* L.) and examined the effect on honeybees feeding on leek nectar. The leek lectin is a monocotyledonous mannose-binding lectin and strongly resembles GNA in terms of its amino acid sequence, sugar specificity and molecular structure. The conclusion of the study was that bees are able to inactivate or degrade the lectin during the honey making process. The ability of bees to survive on leek nectar is important in light of the use of monocotyledonous mannose-binding lectins as resistance factors against sucking insects. The authors felt this conclusion was reassuring and suggested that incorporating a lectin such as GNA into crop plants was unlikely to affect beneficial insects. Researchers have also considered insect predators that feed on insects that have ingested plant material containing GNA. A study conducted by Hogervorst et al. [44] concluded that the GNA protein, when fed directly in a sucrose solution, had an effect on three aphid predators. GNA was found to accumulate in the gut of the insects and to reduce their longevity. However, different research groups have obtained different results depending on the GNA concentration used. In the work by Hogervorst et al. [44], for example, a concentration of 1% GNA in a sucrose solution was used. In contrast to this, Down et al. [45] found no appreciable effect on longevity or fecundity when the two-spot ladybird (*Adalia 2-punctata*) was given aphids that had been fed a diet containing only 0.1% (w/v) GNA. Wakefield et al. [46] used tomato plants expressing 2% GNA and potato plants expressing approximately 0.1% GNA as a food source for the tomato moth, *L. oleracea*. They studied the tomato moth endoparasitoid *Meteorus gyator* and found that there was no significant effect on the endoparasitoid lifecycle and also that they could not detect the GNA protein in adults. Setamou et al. [47] concluded from their laboratory and cage studies that the presence of GNA in sugarcane did not affect the parasitism of sugarcane borers by *Cotesia flavipes*. In the work of Shah et al. [34], it was determined that GNA would not compromise the use of the fungus *Pandora neoaphidis* as a biocontrol agent against the cereal aphid *Metopolophium dirhodum*.

It would be interesting to conduct a field trial using GNA-expressing papaya plants and monitor non-target insects. Given the low levels of GNA protein expressed in the papaya tissue it



seems likely that there should not be any deleterious effects on non-target insects.

Future efforts will determine the inheritance of the GNA transgene in progeny and their reaction to insect predators under field conditions. The inheritance of GNA over multiple generations in rice was studied by Li et al. [40]. They found that the GNA gene was inherited in progeny and that these plants showed improved resistance to the brown planthopper and rice leaf roller in a laboratory assay. Sun et al. [41] examined transgenic rice to a 6th generation, demonstrating GNA expression comparable to parent plants and resistance to the small brown planthopper. As the recombinant GNA is under the control of a constitutive promoter it would also be interesting to examine the level of GNA expression in papaya fruit.

It appears from the present work that incorporating a lectin into papaya has afforded partial resistance against the carmine spider mite by somehow lowering the mites' reproductive capacity. Our laboratory observations are that mites spend less time feeding on leaves expressing GNA than when feeding on control leaves not expressing GNA. A simple analysis indicates that the differences in mite feeding behavior caused by GNA expression may be as significant as is the insecticidal activity of the protein. If each parental female has the same inherent ability to lay eggs and the time taken for eggs to hatch is also a constant, then any changes in the reproductive capacity of the adult mites can be attributed to their feeding behavior. It can be concluded that feeding on GNA-containing leaves is having an adverse effect on the number of eggs produced. In fact fecundity reduces proportionately as concentration of lectin increases. The difference in the number of hatched juveniles can be explained by a delay in egg laying. On the Kapoho leaves, the mites spend most time feeding and lay eggs later than on the transformed lines. Juvenile mites were seen on T8, T12 and T16 as the parent mites spend more time wandering over the leaf surface and laid eggs earlier than the ones on kapoho. It is interesting to note the mites feeding on the line with the highest level of GNA, T35, not only laid the lowest number of eggs but there must also have been a delay in their laying as none of the eggs had hatched at 1 week.

Future experiments will investigate the resistance of the transgenic papaya plants to attack by other pathogens and consideration will be given to the impact of GNA-expressing papaya plants on the natural flora and fauna found in the fields in Hawaii.

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